Development and characterization of genipin crosslinked gelatin emulsion hydrogels and gelatin-starch inclusion physical hydrogels

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This is to certify that the thesis entitled "Development and characterization of genipin crosslinked gelatin emulsion hydrogels and gelatin-starch inclusion physical hydrogels" submitted by **Mr. Sarada Prasanna Mallick** in partial fulfillment of the requirements for the award of Master of Technology Degree in **"Biotechnology"** at the National Institute of Technology, Rourkela, Odisha is an authentic work carried out by him under my supervision and guidance. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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Date: ***Sarada Prasanna Mallick***

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ABBREVIATIONS

Abbreviation	Definitions
EHs	Emulsion Hydrogels
uEHs	Uncrosslinked Emulsion Hydrogels
cEHs	Crosslinked Emulsion Hydrogels
PH	Physical hydrogels
CF	Ciprofloxacin
MZ	Metronidazole
CPDR	Cumulative percent drug release
MO	Mustard oil
GS	Gelatin Solution
DW	Distilled Water
w/w	Weight by Weight
w/v	Weight by Volume
T_m	Melting Point
SS	Stainless Steel
µm	Micrometer
R^2	Regression coefficients
SR	Swelling Ratio
OD	Optical Density
CS	Corn Starch
SS	Soluble Starch
BS	Boiled Starch

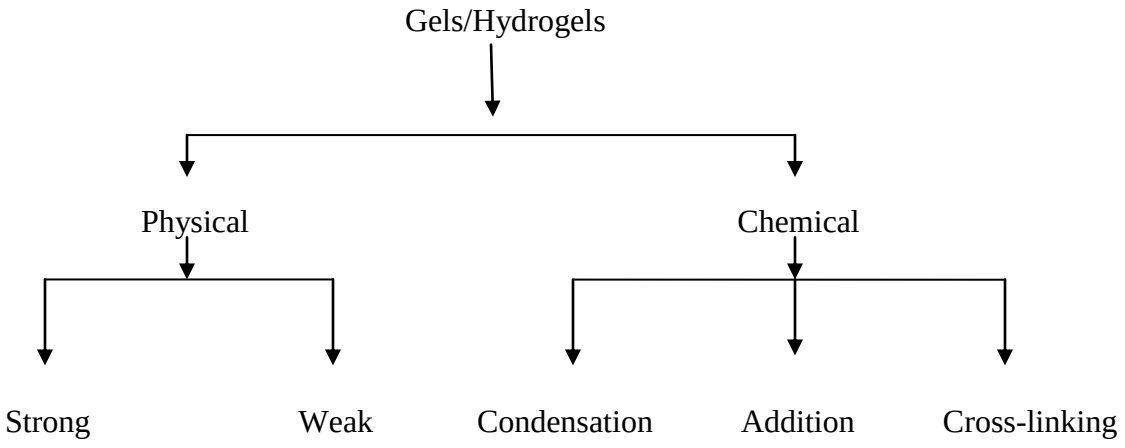
REVIEW OF LITERATURE

1. HYDROGELS

Gels are semisolid, viscoelastic systems containing dispersion of inorganic or organic molecule which entraps the solvent mobility.

Hydrogel is defined as the water swollen colloidal gel in which the liquid ingredient is water. These are three dimensional structures in which the polymer gets swollen upon imbibitions of large amount of water. Hydrogel allows free diffusion of some molecules and the polymer act as a matrix to hold water together. Hydrogels can absorb water or other biofluids with some being able to swell[1]. The property of hydrogel includes pore size, fabrication techniques, shape and surface/volume ratio, H_2O content, strength and swelling activation. Due to significant water content in the hydrogel structure it shows the degree of flexibility.

Hydrogels are having different form of structure[2]. Hydrogels structures are of three types macroporous, microporous and non-porous[3].



There are two types of hydrogels physical and chemical.

1.1. Physical Hydrogel

Physical hydrogels (PHs) are thermoreversible gel networks made of molecular rearrangement and secondary forces including hydrophobic interactions. It is categorized in to two types strong and weak. Strong gel includes glassy nodules, lamellar microcrystals and double/triple helices. Example includes elastomers / block copolymers and gelatin. Weak gels are due to hydrogen bonds, ionic and hydrophobic associations. Example includes xanthan, paint and matured acacia gum etc[4-5]

1.2. Chemical Hydrogel

When the gels are covalently cross-linked it is called as chemical hydrogel. They are also termed as permanent hydrogel. The crosslinking density and the polymer-water interface are responsible for maintaining the swelling state symmetry of the hydrogels. Chemical hydrogel can be formed by the addition of critical percolation for example polyester gel, also from condensation for example polyester gel and form crosslinking[6-7].

2. CLASSIFICATION OF HYDROGELS

There are generally two types of hydrogels.

a. Natural hydrogels

b. Synthetic hydrogels

2. a. Natural hydrogels

Natural hydrogels are existing naturally in the environment. These are the material being inspected for articular tissue engineering. The polymer used in the natural hydrogels are natural

hydrogels are gelatin, methyl cellulose, alginate, agarose/agar, fibrin, chitosan, hyaluronan, chondroitin sulfate and other naturally derived polymers.

Advantages

Due to the excellent biocompatibility natural hydrogels used for tissue engineering application. Natural hydrogels are also having different application like they are low poisonous byproducts, intrinsic cellular interactions and biodegradable in nature.

Disadvantages

The negative aspect of natural hydrogel includes variation of batch, low mechanical strength and the material derived from animal may pass on viruses.

2. b. Synthetic hydrogels

Synthetic polymer hydrogels constitute a group of materials used in numerous biomedical disciplines and developing for new promising applications. These hydrogels are synthesized artificially. Manufacture microarrays and soft contact lens are produce from synthetic hydrogel polymers. Synthetic hydrogels have excellent mechanical properties. Synthetic hydrogels are made from protein-polymer adducts. The synthesis of hydrogels was performed through radical copolymerization. For some cases synthetic hydrogels can perform the task of natural hydrogel[8].The polymer used in the synthetic hydrogels are polyanhydrides, poly(aldehyde guluronate), Poly ethylene glycol etc.

Advantages

Synthetic hydrogels shows various biomedical applications like coating and in devices. These hydrogels are having very low immunogenicity and minimize the risk of biological impurities

Disadvantages

They are having poisonous substances and low degradability.

3. Application of Hydrogels

Hydrogels are utilized naturally by the human body, for example cartilage, mucin, blood clots and vitreous humor of the eye. There is various of application Hydrogels like soft contact lenses, Pills/capsules, Bioadhesive carriers, Implant coatings, etc.

3.1. Application of hydrogels in Drug Delivery Delivery

Hydrogels are having various helpful applications in drug delivery and pharmaceutical sciences due to their large amount of water content. Hydrogels are mainly utilized for conventional controlled drug release system, bioactive materials etc. Hydrogel based drug delivery system can be used for various types like oral, ocular, conventional, epidermal and subcutaneous application. Hydrogels is the suitable medium for the drug delivery due to its biocompatibility, network structure. Hydrogels are also applicable gene delivery and subcutaneous delivery.

3.2. Application of hydrogels in Tissue Engineering

Hydrogels are three dimensional water swollen structures which is insoluble networks of crosslinked hydrophilic polymers. Hydrogels plays an important role in different tissue engineering application. Hydrogels have been used as scaffold materials for various purposes like tissue replacement, drug delivery, call and tissue delivery, bioactive molecule delivery, space filling agent and various other applications[9].

3.3. Application of hydrogels in Biomedical Engineering

Hydrogels have been effectively used in various biomedical applications due to its biocompatible and biodegradable nature[2, 10]. For the biomedical application most of the polymer used for cytotoxicity and in-vivo toxicity tests. The application of hydrogel in biomedical area contains Phospholipids bilayer, energy conversion system, mass transport properties etc.

3.4. Application of hydrogels in Biomaterials

Hydrogels have been used in various applications in biomaterials due to the biodegradable and bioadhesive nature. The example includes soft contact lenses, wound dressing and superabsorbent.

3.5. Application of hydrogels in Agriculture

Hydrogel have been used in various agricultural applications. The water holding capability of the soil increased when the hydrogels are added to the surface. It also decreases the nutrient loss from the soil. Hydrogels are less efficient in the saline soil. Hydrogels can be applied directly in the soil or by spraying.

4. Limitations

The limitations of hydrogels includes high cost, Low mechanical strength, Difficult to load, Difficult to sterilize, can be hard to handle and Non adherent in nature. The limitation of using hydrogels for engineering tissue is poor in mechanical characteristics. Due to the low tensile potency many hydrogels limit their use in load bearing application and can result in flow away of hydrogel from a targeted local site[11-12].

5. Gelatin

Gelatin can be obtained from collagen of bones, ligaments and tendons. Due to the gelling agent gelatin can be used in various purposes like in pharmaceutical industry, food industry etc. Gelatin is having various application in food, biomedical and nutritional properties. Gelatin can be extracted from two processes acid process and alkaline process.

5.1. Use of Gelatin

The functional uses of gelatin include Stabilizer, gelling agent, emulsifying agent and crystallization inhibitor. Gelatin in a highly purified form is a fascinating substance for food and recognized to have thickening, jellifying, foaming, viscosity enhancing, binding, emulsifying, filming etc. It is used for its pharmaceutical, photographic and technical applications.

The pharmaceutical industry uses very large quantities of gelatin for making hard and soft gel capsules.

There are two types of gelatin Edible (e.g. Jello (gelatin + sucrose + flavor) & Knox (plain gelatin)) and Inedible (e.g. Glue, paste)

5.2. Technical use

Gelatin are having various technical use like in coating and sizing, Paper Manufacture, Printing Processes, Protective colloidal applications, matches, Coated Abrasives, Adhesives, Gelatin Films and Light Filters, Microencapsulation etc.

6. Application of Gelatin

6.1. Food Industry

It plays an important role in the food industry.Gelatin is also used as a binding and glazing agent in meat and aspics[13].

5.2. Pharmaceutical Health Industry

In the pharmaceutical health industry, gelatin is highly digestible.

5.3. Cosmeceuticals

Gelatin has been used for many years in the cosmetics industry as in shampoos, conditioners and lipsticks etc.

6. Manufacture of empty gelatin capsules

Steps involving in making gelatin capsules are Dipping, Spinning, Drying, Stripping, Trimming and joining and Polishing.

Crosslinkers are either homo or hetero bifunctional reagents with identical or non identical reactive groups. They can be covalent bonds or ionic bonds.

7. Genipin

Genipin can be obtained from an iridoid glucoside, geniposide abundantly present in the fruit of *Genipa Americana* and *Gadenia jasminoides*. *Gadenia jasminoides* is an evergreen flowering plant of the family Rubiaceae. Iridioid compound is a large class of natural products generally present in plants. They are the source of yellow pigments traditionally used in East Asia for

dyeing textiles also edible blue pigment used in the food industry. The blue pigments are important because they are highly stable to heat, pH and light. Genipin was reacted with beta-LG to produce a new class of modified molecules[15]. The structure of the genipin was discovered in 1960's by Djerassi and his colleagues. The molecular formula of genipin is $C_{11}H_{14}O_5$. The molar mass of the genipin is 226.226 g/mol. Genipin and its derivatives have been used as an herbal medicine, biomedical products, tissue engineering application and a natural colorant in the food industry. Genipin has been widely used as antiphlogistic and a cholagogue in herbal medicine. Genipin is less toxic and degrades more slowly as compared to formaldehyde and glutaraldehyde. It has been reported that genipin bind with biopolymer like chitosan and gelatin. A dark blue color look was found when genipin crosslinked with gelatin. Time and polymer/genipin concentrations are the main two factors in genipin crosslinking reaction. Genipin also used in Cell encapsulation. Genipin is sparingly soluble in aqueous buffers. Genipin is being examined as new way of latent fingerprints on paper products forensic science. Recently genipin has come to attention in biomaterial industry. Chitosan and gelatin crosslinked with genipin has been reported to increase neuroblastoma cell adhesion and its proliferation. Genipin can be used as regulating agent in drug delivery. For the stability of genipin thin layer chromatography was used. Genipin exhibited significant topical anti-inflammatory effect and anti-angiogenic properties.

Glutaraldehyde (GA) is an organic compound having chemical formula $CH_2(CH_2CHO)_2$. GA is a pungent colorless oily liquid. GA is an aliphatic dialdehyde that undergoes most of the typical aldehyde reactions to form acetals, cyanohydrins, oximes, hydrazones and bisulfite complex.GA has found spread use for enzyme immobilization[16].

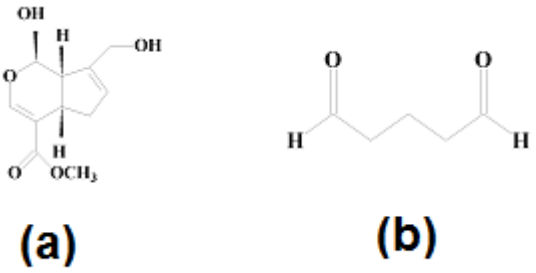


Fig 3.(a) Genipin (b) Glutaraldehyde

8.Emulsion

Emulsion are defined as a thermodynamically unstable system which consist of two immiscible liquid phases which are converted in to a single liquid phase in the help of emulsifying agent. Emulsion can be delivered by oral, topical and parenteral routes.

8.1.Water in oil emulsion (w/o)

In the water in oil emulsion oil is the dispersion medium where the water is dispersed phase. In this process it is not depends on the water and oil ration rather depends on the type of emulsifier.Due to better water resistant it is used as the sun protective factor. It is generally choose for external use like cream.

8.2. Oil in water emulsion (o/w)

In the oil in water emulsion water is the dispersion medium where the oil is the dispersed phase.It is generally oily and not water soluble. Oil in water emulsion are having various use like

they are used in the adjuvants for the influenza vaccines. It is usually prefered because because it provide cooling when used externally for e.g. vanishing cream.

8.3. Dilution Test

It can be of two type's o/w emulsion diluted with water and w/o emulsion diluted with oil.

8.4. Conductivity Test

Continuous phase of water is greater than continuous phase in oil.

8.5. Dye-Solubility Test

Water soluble dye will dissolve in the aqueous phase. The oil soluble dye will dissolve in the oil phase.

9. Application of Emulsion

There are several of applications are there for emulsion like oily drugs are prepared in form of emulsion. In the metallurgical method the concentration of ore by forth floatation process is based upon the treatment of the powdered ore in the emulsion. Milk is an emulsion of liquid fats in water. The cleaning action is based upon the formation of oil-in-water emulsion.

10. Starch

Starch is a natural, low-priced, obtainable, renewable, and biodegradable polymer produced by many plants as a source of stored energy. It is the most rich storage polysaccharide in plants and

described as, pulse and tubers. A glycosidic bond joins the glucose moieties of starch. Amylase and amylopectin are the two main composition of starch [17].

10.1. Amylose

Amylase is a linear polymer generally made of D-glucose unit. This polysaccharide is one of the two main components of starch. Which make up 20-30% of the structure. Amylose normally contributes to gelling characterstics.

10.2. Amylopectin

Amylopectin is a soluble polysaccharide and highly branched polymer of glucose found in plants. Glucose units are linked with α glycosidic bond. It formulates 70-80% of the structure. Amylopectin normally contributes to thickening agent.

Corn Starch

Corn starch is obtained from the corn (maize grain). The corn plant converted large amount of radiant energy in to stable chemical energy. Corn starch is having various uses like thickening agent in foods for e.g. soup and sauces etc.

Soluble starch

Soluble starch is defined as a high molecular weight water soluble dextrin manufactured by partial hydrolysis of starch. It is white amorphous powder. It is quite dispersible in hot water.

Use of Starch

Starch is having several of use like in Beverages, Confectionery, Bakery products, Chocolate, Processed foods, Desserts & Dairy, Paper & Board, Pharmaceutical & Cosmetics, Aqua feed, Animal feed, Pet food and in various industrial applications.

16. Application of starch

Starch and its modified counter parts have found its wider applicability in food and non-food modified starches have a wide variety of use both in the food and non food vicinities. The major application of starches lies in pharmaceuticals, food and cosmetics industry. Starch plays an increasing role in the field of biodegradable plastics, packaging material, printed circuit boards, dry cell batteries and moulds. Starch is used as an adhesive for example hot-melt glues, stamps, bookbinding, envelopes, wood adhesive, lamination, automotive, corrugation and paper sacks[18].

Development and characterization of genipin crosslinked gelatin emulsion hydrogels

CHAPTER 1

Abstract

The present study discusses about the development and characterization of genipin-crosslinked gelatin based emulsion hydrogels (EHs). EHs were prepared by varying the proportions of gelatin solution and mustard oil. Both the uncrosslinked (uEHs) and the crosslinked (cEHs) gels were characterized thoroughly by microscopy. The microscopic results suggested that the internal phase droplet size distribution was broader for gels with higher proportions of oil. Thermal properties of the gels were found to be affected by both gelatin and mustard oil proportions. The gels were loaded with ciprofloxacin (CF, model drug). The pHs of the gels were within the limits of the pH of the human skin. The gels were hemocompatible and tried as a carrier for controlled drug delivery.

1. Introduction

Gels are defined as 3D polymeric networked structures having the ability to undergo extensive swelling when immersed in proper solvent [19-22]. If the absorbed solvent is aqueous in nature, the gel is regarded as hydrogel. The swelling properties of the hydrogels may be tailored by changing the crosslinking density of the polymeric matrix. Since the diffusion of the drug molecules are dependent on the amount of physiological solution present within the matrices, altering the crosslinking density of the matrix has been found to modulate the release properties of the bioactive agents [23]. Various biopolymers (e.g. gelatin, chitosan, alginate, celluloses and collagen) have been used for the designing of the hydrogels for pharmaceutical applications [23-30]. Gelatin is a protein based biopolymer, which is derived from animal collagen. It is a highly biocompatible biopolymer and hence has been used in the development of various pharmaceutical products [23, 31-33].

Mustard oil (MO) MO has been traditionally extensively used in the southern Asian countries (India, Pakistan and Bangladesh) for the skin massages of infants and adults [34-36]. Apart from this, MO containing emulsions have been developed for the topical ocular delivery of non-steroidal anti-inflammatory drugs (NSAIDs) *viz.*, diclofenac [37]. In recent years, MO has been used as an adjuvant for the development of novel new nutritive-immune enhancing delivery system [38-39]. Taking inspiration from the above studies, MO was used as the representative oil.

In the current study, attempts were made to develop genipin crosslinked mucoadhesive gelatin EHs as a matrix for controlled delivery of antimicrobials. EHs may be defined as the hydrogel based matrix system in which an oil has been distributed uniformly [40-41]. Genipin is a naturally occurring crosslinker obtained from the fruits of *Gardenia jasminoides*. It has been

gaining importance in biomedical industries due to its far less cytotoxic nature as compared to glutaraldehyde [42-44]. The developed gelatin based EHs were studied for their suitability as dermal/transdermal drug delivery systems.

2. Materials and methods

2.1. Materials

Tween 80 (polyxyethylene sorbitan monooleate), gelatin and glycine were procured from Himedia, Mumbai, India. Ciprofloxacin (CF) was procured from Fluka Biochemica, China. Sodium citrate was procured from Loba Chemie, Mumbai, India. Genipin was procured from Challenge Bioproduct Company Limited, China. Mustard oil (MO) was purchased from local market. Goat intestine and blood were collected from the local butcher shop. Double distilled water (DW) was used throughout the study.

2.2. Preparation of EHs

The EHs were prepared as per the method reported earlier with slight modifications in the procedure [40]. In short, twenty percent (w/w) gelatin solution was prepared by dissolving 20 g of gelatin in 80 g of DW, kept on stirring at 50 °C (GS). To this clear homogenous solution, 2 ml of Tween 80 was added. MO, maintained at 50 °C, was added to GS and homogenized at 800 rpm for 15 min to form primary emulsion (PE). 0.1 g of genipin was added to the PE and further homogenized for 30 sec. The emulsion was subsequently poured into petri-plates and incubated at 40 ^{o}C for 30 min to promote crosslinking of the gelatin matrix. Excess genipin was neutralized by 1% (w/w) glycine solution [22]. The crosslinked gels were washed thoroughly with DW and stored under refrigerated conditions (5 °C). These gels were regarded as cEHs. CF loaded EHs were prepared in a similar manner by using 1% (w/v) CF solution in MO as the internal phase. In

the similar manner, uncrosslinked EHs (uEHs) were prepared by pouring the PEs into perti-plates and subsequent incubation at 4 °C for 30 min. The gels were stored under refrigerated conditions for further analysis.

2.3. Microscopic evaluation of the gels

The microstructures of the molten uEH gels were studied under compound bright-field microscope (Leica-DM750 equipped with ICC 50-HD camera, Germany). The droplet size distribution of the emulsions was analyzed using NI vision assistant-2010 (USA) software as per the reported literature [45].

2.4. Thermal studies

The melting points of the uncrosslinked gels (uEHs) were analyzed by falling ball method, as per the reported method [46]. In short, a stainless steel (SS) ball (diameter 1/8 inch and weight 130 mg) was put over the 2 g formulation, kept in a 10 ml test-tube under refrigeration. The gels were heated at a rate of 1 °C/min. The temperature at which the SS ball is completely submerged into the formulation was noted as the melting point (T_m) of the gels.

2.5. Swelling behavior

The swelling behaviors of the cEHs were determined as per the reported literature. In short, accurately weighed (Wo) gels were incubated in a beaker containing 100 ml of DW at RT [47]. At regular intervals of time (15 min for the first 1h and thereafter 30 min up to 8h), the EHs were taken out. The surface bound water was removed by wiping with a tissue paper. The weights of the cEHs were accurately measured using a digital weighing balance (W_t). Swelling ratio (SR) of the EHs was calculated as per the formula given in equation 1 [47].

$$SR = \frac{(W_t - W_0)}{W_0} \quad (1)$$

Where, SR=swelling ratio

W_t is the weight of the swollen gel at time t.

W_0 is the initial gel weight

2.6. pH measurement

The pHs of the uEHs and cEHs were measured using a digital pH meter (Model 132E, EI products, Mumbai, India) as per the reported literature [48].

2.7. Hemocompatibility test

The hemocompatibility test was done as per the ASTM protocol described in the previous literature [22, 28-29, 31-32, 49-53]. The % hemolysis of the blood was calculated as per the equation 2.

$$\%\ Hemolysis = \frac{OD_{test} - OD_{Negative}}{OD_{positive} - OD_{Negative}} \times 100 \quad (2)$$

Where,

OD_{test} = Absorbance of test sample,

$OD_{positive}$ = Absorbance of positive control and

$OD_{negative}$ = Absorbance of negative control.

2.8. *In vitro* drug release studies

In vitro drug delivery studies of uEHs were performed using modified Franz diffusion cell [54]. 1.5 g (approx) of the uEHs were weighed accurately and kept into the donor chamber of the

diffusion cell. The donor and receptor chambers were separated by a dialysis membrane. The receptor volume was maintained at 50 ml throughout the study. During the study, sampling was done at every 15 min during first hour and subsequently at every 30 min in the next 7h. During each sampling, the whole receptor medium was replaced with the fresh medium (DW). At the end of the study, the samples were analyzed for the presence of CF at 271 nm using UV-visible spectrophotometer (UV-3200, LABINDIA, Mumbai, India).

The drug dissolution tests of the cEHs were carried out in single basket dissolution apparatus for 8 h. Accurately weighed 1.5 g (approx) of EHs were cut. The drug containing EHs were put into the dissolution basket containing 900 ml of DW. The speed of the stirrer was kept at 100 ± 2 rpm and the dissolution medium temperature was maintained at 37 ± 2 °C. 3 ml of samples were withdrawn at regular intervals of time (15 min for the first hour and 30 min for the next 7 h). Fresh dissolution medium of same volume was replaced after each sampling. The samples were analyzed at 271 nm using UV-visible spectrophotometer.

3. Results and discussion

3.1. Preparation of EHs

The EHs were prepared by varying the proportions of gelatin solution and oil. The compositions of the gels have been tabulated in table 1. The blank gelatin gel was light brown in color and was translucent. The uEHs were yellowish in color due to the yellow color of the MO. There was an increase in the yellowish tinge of the uEHs as the proportion of MO was increased in the gels (Fig. 1). On the other hand, cEHs were found to be dark blue color (Fig. 2), a characteristic color produced due to the reaction of the primary amino groups and genipin [55-56]. The color of the cEHs was dependent on the composition of the gels [57]. With the increase in the proportion of the MO in the cEHs, there was subsequent increase in the dark blue color due to higher crosslinking density of the gelatin matrices. Both types of the EHs had a smooth texture and were opaque. There were no changes in the textural properties of the gels (uEHs and cEHs) after the addition of 1 % (w/w) CF drug. All the drug containing formulations were found to be stable.

Table1: Composition of the prepared gels

Samples	Volume of 20 % (w/v) gelatin solution (ml)	Volume of MO (ml)	Genipin (g)	Ciprofloxacin (% w/w)	Result
uG1	20.0	0	--	--	Gel formed
uG2	17.5	2.5	--	--	Gel formed
uG3	15.0	5.0	--	--	Gel formed
uG4	12.5	7.5	--	--	Gel formed
uG5	10.0	10.0	--	--	Gel formed
cG1	20.0	0	0.1	--	Gel formed

cG2	17.5	2.5	0.1	--	Gel formed
cG3	15.0	5.0	0.1	--	Gel formed
cG4	12.5	7.5	0.1	--	Gel formed
cG5	10.0	10.0	0.1	--	Gel formed
uG1D	20.0	0	--	1.0	Gel formed
uG2D	17.5	2.5	--	1.0	Gel formed
uG3D	15.0	5.0	--	1.0	Gel formed
uG4D	12.5	7.5	--	1.0	Gel formed
uG5D	10.0	10.0	--	1.0	Gel formed
cG1D	20.0	0	0.1	1.0	Gel formed
cG2D	17.5	2.5	0.1	1.0	Gel formed
cG3D	15.0	5.0	0.1	1.0	Gel formed
cG4D	12.5	7.5	0.1	1.0	Gel formed
cG5D	10.0	10.0	0.1	1.0	Gel formed

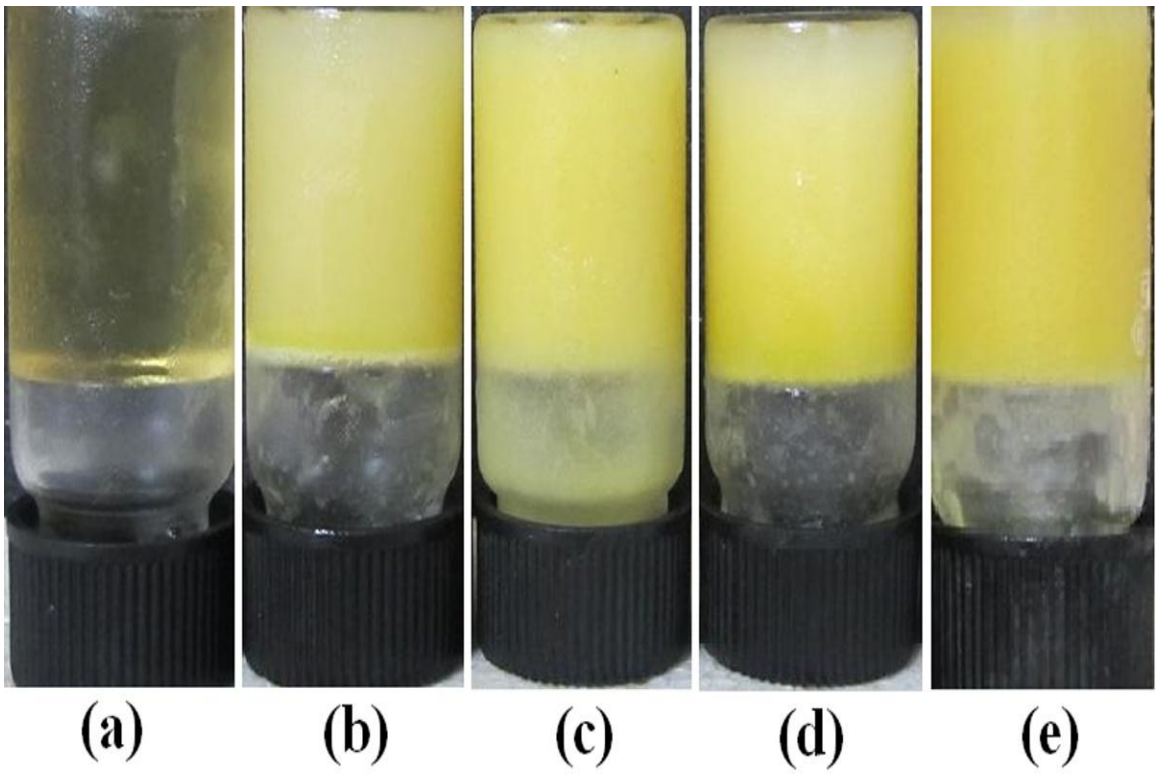


Fig. 1: uEHs of different compositions (a) uG1, (b) uG2, (c) uG3, (d) uG4 and (e) uG5

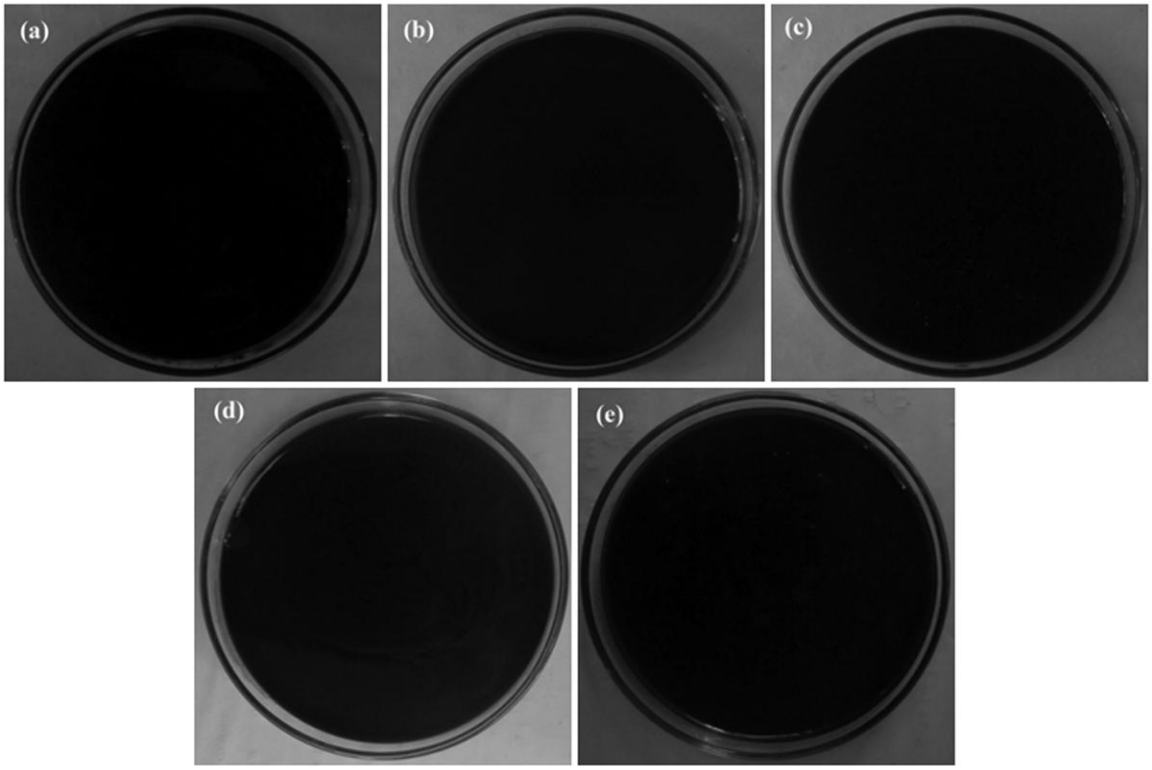


Fig. 2: cEHs of different compositions (a) cG1, (b) cG2, (c) cG3, (d) cG4 and (e) cG5

3.2. Microscopic evaluation of the emulsions

The micrographs of the molten uEHs have been shown in Fig. 3. The micrographs suggested the presence of dispersed circular MO droplets within the GS continuum phase. There was an increase in the size of the dispersed phase droplets with the increase in the MO proportion. The droplet size distribution of the internal phase in the gels followed Gaussian distribution (Fig. 4a). This type of distribution is achieved when physical methods are used for the preparation of the emulsions [58-59]. The sizes of the droplets were in the range of 10-30 μm. 50 % of the droplet's

population was having the size of 15 microns (approx) (Fig. 4b). The presence of narrow size distribution gives an indication of a probable stable emulsion [60].

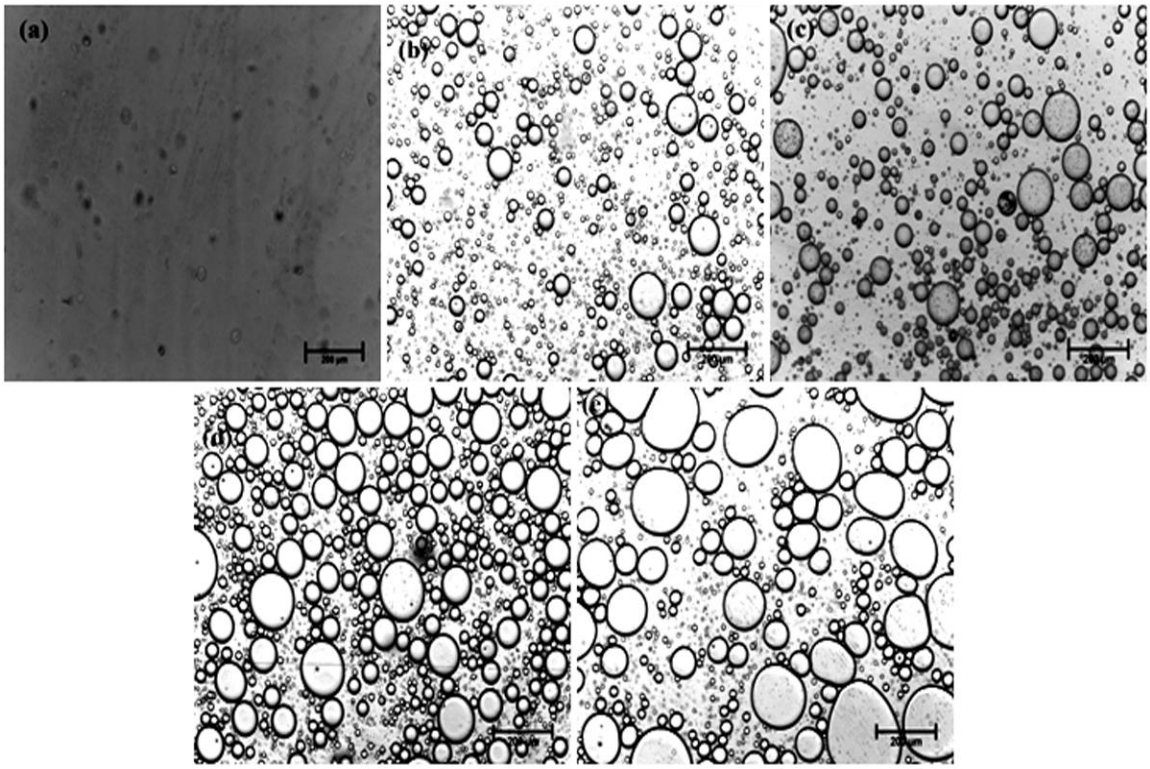


Fig. 3: Light micrographs of MO-in-gelatin sol emulsion. (a) uG1, (b) uG2, (c) uG3, (d) uG4 and (e) uG5 gels.

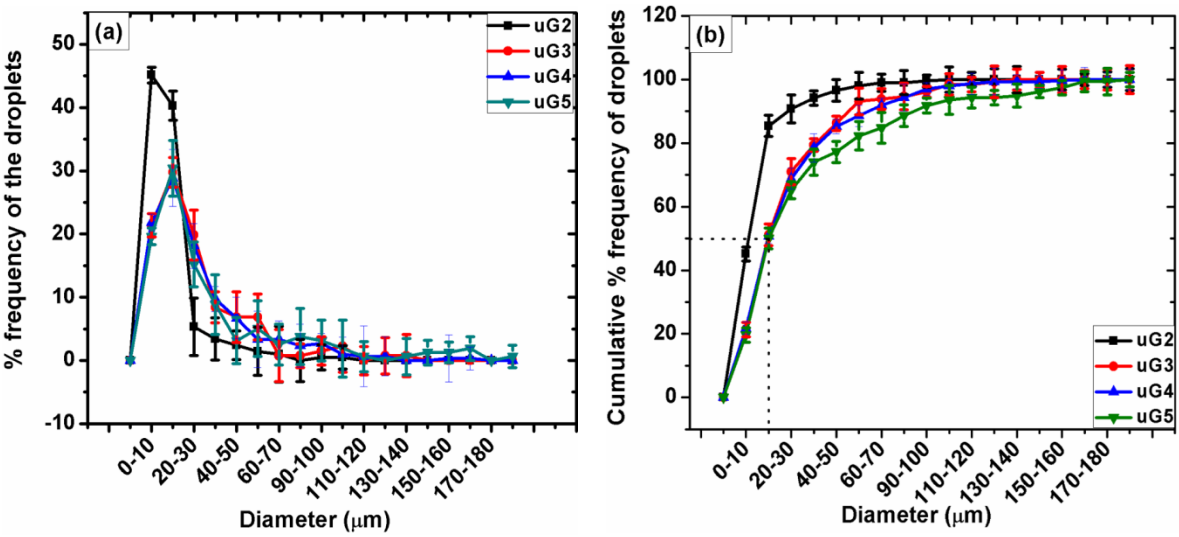


Fig. 4: Droplets size distribution of uEHs, in terms of their (a) % frequency and (b) cumulative % frequency

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3.3 Thermal studies

The melting point (T_m) of the uEHs was determined by drop ball method as reported earlier (table 2) [46]. The T_m of the gels was found to be decreasing as the proportion of MO was increased. Higher T_m of uG1 may be due to the formation of strong physical polymeric network when gelatin molecules were dissolved in water. Incorporation of MO within the gelled structure resulted in the reduction of the intensity of the intermolecular hydrogen bonding which, in turn, caused reduction in the T_m.

Table 2: The melting point values of uEHs by drop ball method

Samples	T_m (°C)
uG1	32.10 ± 2.66
uG2	31.20 ± 1.92

uG3	30.90 ± 2.44
uG4	30.50 ± 3.32
uG5	30.00 ± 2.26

3.4. Swelling studies

The swelling behaviour of the crosslinked gels have been shown in Fig. 5. The swelling behaviour was dependent on the MO content in the gels. The amount and rate of swelling of the gels were lower in gels with higher proportions of MO. This may be attributed to the lower amount of gelatin, which is responsible for the absorption of water. The structural integrities of the gels were intact during the swelling study.

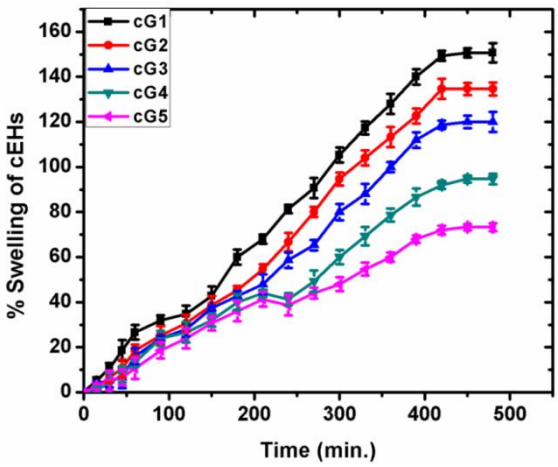

Fig. 5: Swelling behavior of cEHs

3.5. pH measurement

The study of the pH of the pharmaceutical formulation is an important parameter. Various pharmacopoeia have set standards for the pharmaceutical formulations [61]. This is due to the reason that the formulations are meant to be in contact with the cells and tissues. Higher or lower pH values may cause irritation of chemical burn. The results of the study have been tabulated in

table 3. The pH of the EHs was found to be in the range of 5.5 to 7.0. This suggested that the developed gels may be used for transdermal and topical formulations.

Table 3: The pH of hydrogels

Samples	pH
uGH1	5.60 ± 0.75
uGH2	5.62 ± 0.81
uGH3	6.00 ± 0.44
uGH4	7.27 ± 0.82
uGH5	5.80 ± 0.44
cGH1	6.54 ± 0.23
cGH2	6.81 ± 0.45
cGH3	6.43 ± 0.62
cGH4	6.75 ± 0.31
cGH5	6.62 ± 0.13

3.6. Hemocompatibility studies

The % hemolysis of the goat blood in the presence of the leachants of the EHs was found to be below 5% (table 4) suggesting that the gels may be regarded as biocompatibile [62]. Henceforth, EHs may be tried as drug delivery vehicles.

Table 4: % hemolysis of the EHs

Samples	% Hemolysis
uG1	2.60 ± 0.55
uG2	2.19 ± 0.82
uG3	1.10 ± 0.62
uG4	0.68 ± 0.47
uG5	0.41 ± 0.79
cG1	3.82 ± 0.32
cG2	2.86 ± 0.65
cG3	1.50 ± 0.49
cG4	1.36 ± 0.19
cG5	0.68 ± 0.67

3.7. *In vitro* drug release studies

Fig. 6 shows the drug release profile of CF from uEHs and cEHs. The rate of drug release was found to be dependent on the physicochemical properties of the gels (e.g crosslinking density, % swelling and impedance). The higher amount of drug release may be associated with gels with higher % swelling and lower impedance. Higher % swelling leads to higher partition of the drug from gel matrix into the external aqueous phase. This resulted in higher CPDR value from the cG1 than the cG3 and cG5. This may be due to the partition coefficient effect of the drug, which states that the solute distributes itself amongst the two immiscible liquids in a definite concentration ratio [63-65]. Crosslinking of the gels decreased the net amount of drug release.

uEHs have higher cumulative % drug release (CPDR) than the cEHs. The lower CPDR from cEHs might be due to the hindrance of free movement of water within the cEHs.

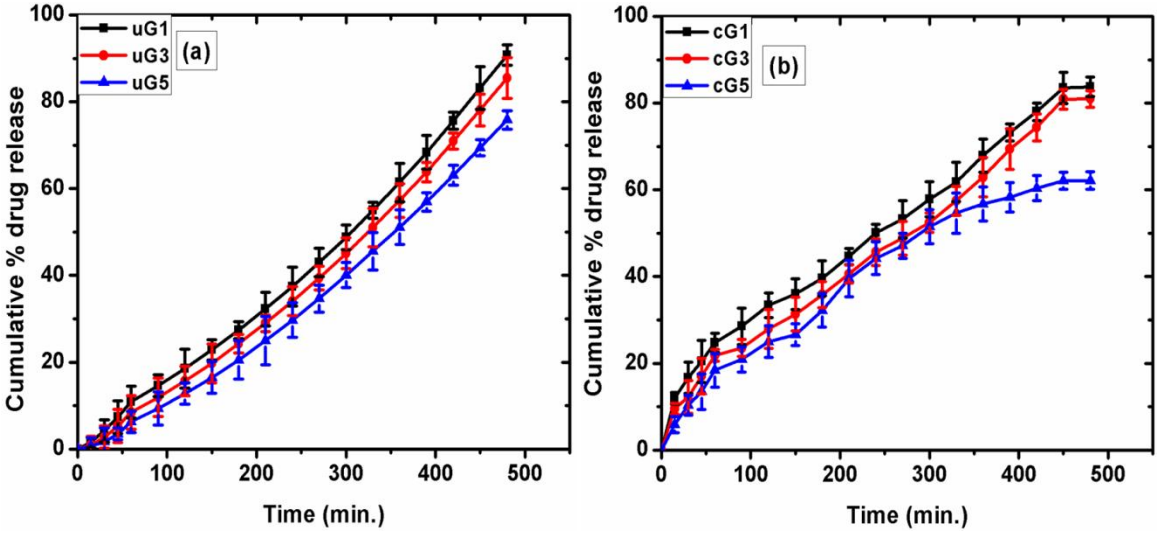

Fig. 6: *In vitro* CPDR profile of CF from (a) uEHs and (b) cEHs

4. Conclusion

The study reports the successful development of a novel gelatin-based EHs using genipin as crosslinking agent. MO was used as representative oil. The effect of proportion of internal oil phase on the properties of the gels was studied by various physical techniques. The amount of drug released was dependent on the oil proportion of the gels. The study suggested that the rate of release of the drugs may be altered by altering the proportion of the internal oil phase. The gels were found to be biocompatible and may be used as matrices for the controlled drug delivery.

Development and characterization of gelatin-starch inclusion physical hydrogels

CHAPTER 2

ABSTRACT

The current study deals about the development and characterization of gelatin-starch based phase-separated hydrogels. The hydrogels were prepared using corn starch, soluble starch and boiled starch. The hydrogels were characterized by pH and thermal studies, Metronidazole (MZ), a model antimicrobial drug, was incorporated within the hydrogels. The hydrogels were highly hemocompatible in nature. All the samples were found to have pH in the range of 5.00-6.00. The Based on the preliminary results, it was concluded that the developed gels have a good potential to be used as carriers for bioactive agents.

1. Introduction

Hydrogels are three-dimensional cross-linked networks of hydrophilic polymers [27, 66]. Due to this, hydrogels are able to imbibe and hold water within its structure, which in turn, results in the migration of water into the core of the polymeric construct. If drug is incorporated into the polymer construct, the absorption of water results in the dissolution of the drug molecules thereby creating a concentration gradient. This results in the diffusion of the drug molecules out of the polymer matrix. The rate of diffusion of the drug out of the hydrogel is dependent on the physical and chemical properties of the hydrogels. Depending on the nature of the hydrogels and how it alters the drug release profile, the hydrogels may be used either as matrices for controlled release or quick release systems [3, 40, 67]. Apart from the pharmaceutical industries, hydrogels have also been used extensively in food [68] and biomedical [69-71] industries. The wide spread usage of hydrogels is mainly due to their inherent biocompatible nature [72]. They may be made biodegradable, designed to support cellular activities [73] and protect cells [74]. The natural polymers used for the design/construction of hydrogels can be broadly categorized either as proteins (e.g. collagen [75] and gelatin [76]) or as polysaccharides (e.g. starch [77], alginate and agarose [78]).

The crosslinking of the polymers to form hydrogels may either be due to physical interactions or chemical bond formation. Physical interactions include ionic crosslinking, hydrogen bonding and molecular entanglement, i.e. there is no formation of covalent bonds. Chemical crosslinking includes formation of covalent bonds during the formation of hydrogels [79]. The mechanical and thermal properties of the chemically crosslinked hydrogels are far better than the physically crosslinked hydrogels [79]. The primary disadvantage of the chemically crosslinked hydrogels is the presence of uncrosslinked starting materials which might cause undesirable side effects [79].

Due to this reason, the physically crosslinked hydrogels have been extensively studied in food industries.

Gelatin is a protein based biopolymer and is obtained by the hydrolysis of collagen from animal sources [80]. It is inherently biocompatible and biodegradable in nature [81]. It has been used extensively not only to develop food and pharmaceutical products but also various other products of biomedical importance [2, 40]. Starch, on the other hand, is a polysaccharide biopolymer [82]. Starch is one of the biopolymers of choice in food, pharmaceutical and biomedical industries. This is due to its abundance in nature, safe for human consumption and biodegradability [83]. Gelatin has been reported to form phase-separated hydrogels with polysaccharides like starch and maltodextrin [84-85]. This paper has been designed to study the effect of composition of the gelatin-starch phase-separated systems on the physical properties of the physical hydrogels. Apart from this, effects of different grades of starches (e.g. corn starch (CS), soluble starch (SS) and boiled starch (BS)) on the properties of the hydrogels were studied in-depth.

2. Materials and methods

2.1. Materials

Gelatin, CS, sodium azide, nutrient agar and dialysis tubing (MW cutoff: 60 kDa) were purchased from Himedia, Mumbai, India. SS was purchased from Merck Specialties Private Limited, Mumbai, India. Hydrochloric acid (HCl) and sodium citrate were purchased from Loba Chemie, Mumbai, India. Ethanol was purchased from Changshu Yangyuan Chemical, China. Metronidazole (MZ) was a kind gift from Aarti drugs, Mumbai, India. Fresh goat blood was obtained from the local butcher shop. All the experiments were carried out using double distilled water (DW).

2.2. Preparation of the hydrogels

Preparation of the suspensions and solutions: A clear homogeneous 20 % (w/w) solution of gelatin was prepared by dissolving 20 g of gelatin in 60 g of DW, kept on stirring at 50 °C. After the dissolution of the gelatin in the DW, the final volume was made to 100 g with DW maintained at 50 °C (GS).

Three kinds of starches were used, *viz*. CS, SS and BS, for the development of the hydrogels. 2 % (w/w) CS or SS dispersion was prepared by dispersing 2 g of CS or SS in 98 g of DW. BS dispersion was prepared by heating 2 % (w/w) of CS dispersion in DW at 80 °C for 30 min. The final weight of the suspension was made to 100 g using warm DW and subsequently cooled to room-temperature (RT, 25 °C). The above solutions and suspensions were used for the preparation of the hydrogels.

Preparation of the hydrogels: Physical hydrogels were prepared by varying the proportions of gelatin solution (GS) and starch dispersions. The compositions of the hydrogels have been tabulated in table 1. The hydrogels were prepared by mixing GS and starch dispersions and subsequently homogenizing the dispersion at 500 rpm for 30 min at 40 °C. The homogenized mixture was then poured into culture bottles and then stored under refrigerated conditions for further analysis. Hydrogel made with GS alone served as a control. The MZ-loaded hydrogels were prepared in a similar manner such that the final concentration of the drug in the hydrogel was 1% (w/v). MZ was dissolved in the GS-starch dispersion mixture during homogenization.

Table 1: Composition of Physical hydrogels

Sample	Volume of starch dispersion (ml)				MZ (%, w/v)
	GS	CS	SS	BS	
GH	20.0	0	--	--	--
CS1	16.0	4.0	--	--	--
CS2	12.0	8.0	--	--	--
CS3	8.0	12.0	--	--	--
SS1	16.0	--	4.0	--	--
SS2	12.0	--	8.0	--	--
SS3	8.0	--	12.0	--	--
BS1	16.0	--	--	4.0	--
BS2	12.0	--	--	8.0	--
BS3	8.0	--	--	12.0	--
GHM	20.0	0	--	--	1
CS1M	16.0	4.0	--	--	1
CS2M	12.0	8.0	--	--	1
CS3M	8.0	12.0	--	--	1
SS1M	16.0	--	4.0	--	1
SS2M	12.0	--	8.0	--	1
SS3M	8.0	--	12.0	--	1
BS1M	16.0	--	--	4.0	1

BS2M	12.0	--	--	8.0	1
BS3M	8.0	--	--	12.0	1

2.3. Microscopic studies

The microstructure of the hydrogels was studied using inverted phase contrast (Olympus INVI-TR attached with SONY digital camera EPL-1, USA) and scanning electron microscopy (JEOL, JSM-6390, JAPAN). The inverted phase contrast microscopy was carried out by converting the physical hydrogels into thin smears.

2.4. Thermal studies

The melting point of the hydrogels was determined by drop-ball method as mentioned in the previous literature [46]. In short, 2 g of the hydrogels were melted and poured in 10 ml of test tubes. The test tubes were incubated for 15 min in a laboratory refrigerator, maintained at 4 ±1 °C. After refrigeration, a stainless steel (SS) ball (diameter 1/8 inch; weight 130 mg) was placed gently on top of the hydrogels. The hydrogels were heated at a rate of 1 °C/min in a melting point apparatus. The melting point (T_m) of the hydrogels was noted when the SS ball completely submerges [86].

2.5. pH Measurement

The pH of the hydrogels was measured using a digital pH meter (EI digital pH meter, model no: 112, India) [87].

2.6. Hemocompatibility test

Hemocompatibility of the hydrogels were determined as per the reported literature [88]. The % hemolysis of the blood was calculated as per the equation 1.

$$\%\ Hemolysis = \frac{OD_{test} - OD_{Negative}}{OD_{positive} - OD_{Negative}} \times 100 \quad (1)$$

2.7. *In* vitro drug release

The drug release study was carried out using a 2-compartment modified Franz diffusion cell, as per the reported literature [89]. In short, accurately weighed 1.5 g of the drug loaded hydrogel was loaded in the donor compartment. The donor compartment was lowered towards the receptor compartment so as to ensure that the dialysis membrane (attached with the donor compartment) was in contact with the receptor fluid (50 ml of DW), kept on stirring at 100 rpm and the temperature was maintained at 37 ± 1 °C. The receptor fluid was replaced by fresh DW at regular intervals (15 min during first hour and subsequently after 30 min during the next 7 h). The samples were then analyzed using UV-visible spectrophotometer (UV-3200, LABINDIA, Mumbai, India).

3. Results and discussion

3.1. Preparation of physical hydrogels

The hydrogels were prepared by varying the proportions of GS and starch dispersions. The organoleptic properties of the hydrogels have been tabulated in table 2. GH was transparent and light brown in color. Incorporation of the starch dispersions within the hydrogels resulted in the formation of opaque hydrogels. The colors of the starch incorporated hydrogels were white and the whiteness of the hydrogels was higher in hydrogels with higher proportions of starch (figure 1). The hydrogels had a pleasant odor and were thermo-reversible in nature. They did not show flow when kept under refrigerated conditions (figure 1). The hydrogels with higher proportions of starch showed flow when kept at RT (room-temperature, 25 C). With the further increase in

the proportion of the starch dispersion, there was no formation of the hydrogels. The restrained flow of the hydrogels at lower temperatures may be attributed to the formation of ordered network arrangements of gelatin molecules [90]. This is due to the presence of α-helical structures in gelatin molecules [85]. Incorporation of MZ within the hydrogels neither altered the gelation property nor caused any change in the organoleptic properties.

Table 2: The stability and nature of physical hydrogels

Sample	Result	Color	ODOUR
GH/GHM	Gel formed	Brownish	Pleasant
CS1/CS1M	Gel formed	Cream	Pleasant
CS2/ CS2M	Gel formed	Cream	Pleasant
CS3/ CS3M	Gel formed	White	Pleasant
SS1/ SS1M	Gel formed	Yellow	Pleasant
SS2/ SS2M	Gel formed	Cream	Pleasant
SS3/ SS3M	Gel formed	White	Pleasant
BS1/ BS2M	Gel formed	White	Pleasant
BS2/ BS3M	Gel formed	White	Pleasant

BS3/ BS3M	Gel formed	White	Pleasant

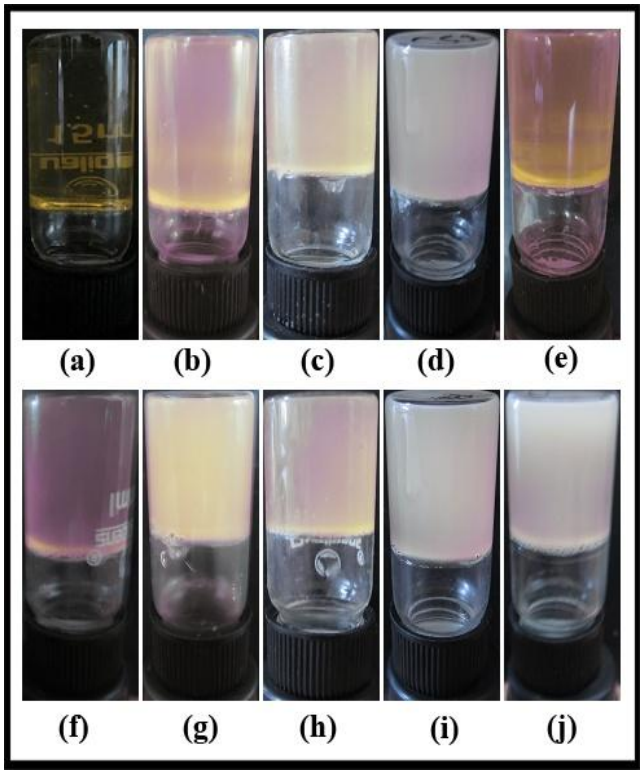

Figure 1: The stable physical hydrogels (a) GH, (b) CS1, (c) CS2 , (d) CS3, (e) SS1 (f) SS2, (g) SS3, (h) BS1, (i) BS2 and (j) BS3.

3.2. Hydrogel morphology

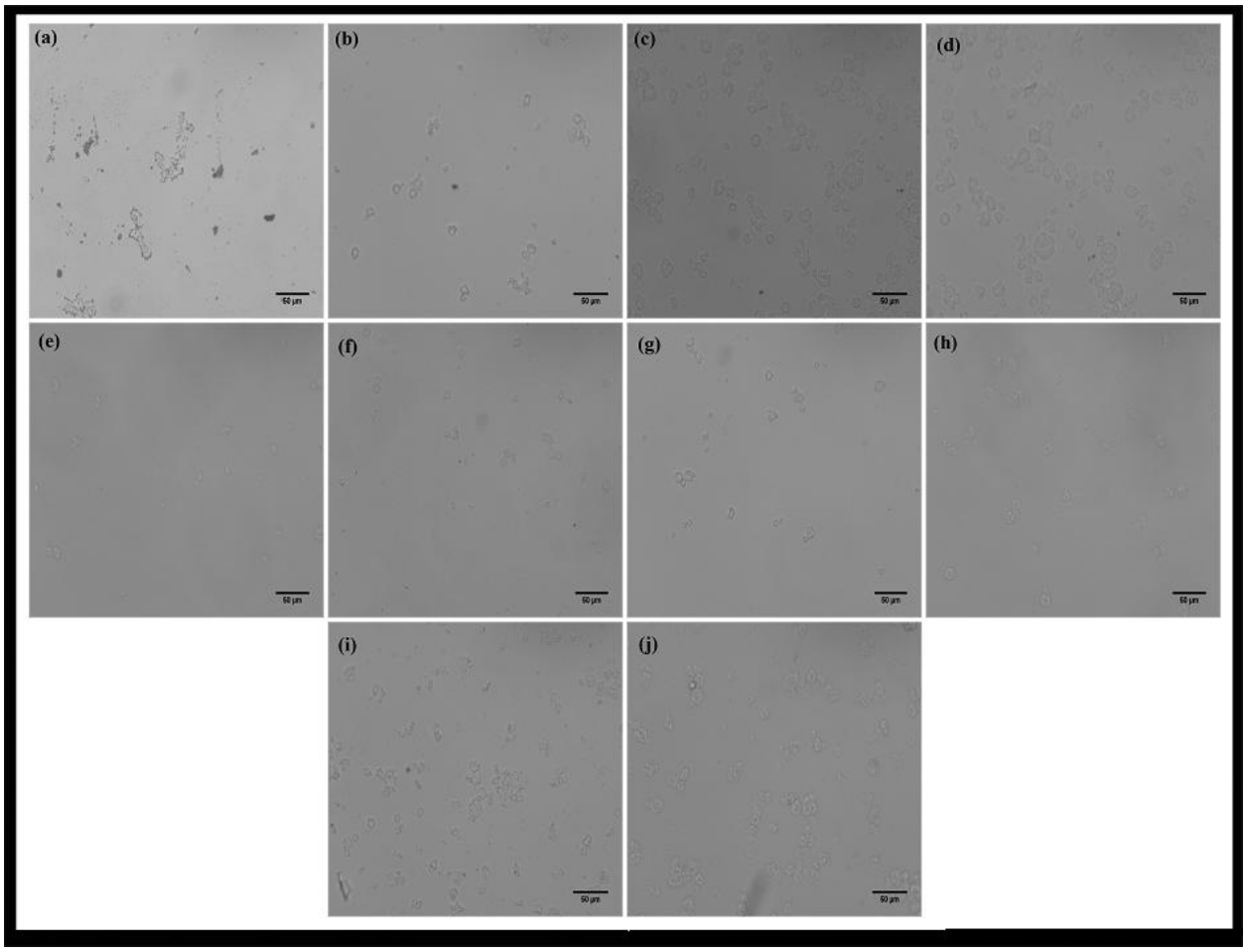

Figure 2: Phase contrst micrographs of hydrogels (a) GH, (b) CS1, (c) CS2 , (d) CS3, (e) SS1 (f) SS2, (g) SS3, (h) BS1, (i) BS2 and (j) BS3.

The microstructures of the hydrogels as visualized under inverted phase contrast microscope have been shown in figure 2. The microstructures of the hydrogels have shown the presence of phase separated starch inclusions in the form of dark globules in the gelatin continuous matrices . The increase in the density of starch inclusions was observed with the increase in starch concentrations. This suggested that the phase separation phenomenon was dependent on the composition of the hydrogels. An increase in the tendency of the phase separation was observed

with the increase in starch concentration. In general, phase separation is a common phenomenon occuring in protein-polysacharide mixtures. Since both gelatin and starch molecules are not polyelectrolytes, there are chances of thermodynamic incompatibility which might resulted in the segregative phase separation [91]. This might have resulted in the increased self-association of the biopolymers [92]. Phase separation has been found to be dependent on the structure and the composition of the biopolymers used.

SS is rich in amylodextrin (linear short chained amylose units) and is devoid of amylopectin. The thermal incompatiblity was found to be lower when linear chained carboxyl containing polysaccharides were used in the protein-polysaccharide system [91]. Similar results were obtained when SS was used for the development of the hydrogels, *i.e.* SS hydrogels showed small starch droplets/inclusions as compared to the CS and BS hydrogels . The presence of larger droplets/inclusions in CS and BS hydrogels may be associated with the presence of hyper-branched polysaccharide units (amylopectin), which lead to the aggregation of polymeric chains [93].

3.3. Thermal studies

The gel-to-sol transition temperature (T_{gs}) was determined by drop ball method. GH showed highest T_{gs} compared to the other hydrogels. This may be due to the presence of only gelatin molecules in GH. With the increase in the concentration of the SS, CS and BS, there was a decrease in the T_{gs} of all the gels. The CS and SS containing gels have shown similar T_{gs}. The T_{gs} of the BS hydrogels were lower than the CS and the SS hydrogels. This can be attributed to the reduction in the proportion of the gelatin, the biopolymer responsible for incorporating

mechanical strength in the hydrogels. The lower T_{gs} of the BS hydrogels might be attributed to the disruption of starch complexes when heated at higher temperatures. This results in the random orientation of the starch molecules [94].

Table 3: The T_{gs} values of the physical hydrogels

Sample	T_{gs} (°C)
GH	35.0 ± 1.5
CS1	32.0 ± 3.5
CS2	28.0 ± 1.5
CS3	23.0 ± 1.0
SS1	33.0 ± 2.5
SS2	25.0 ± 3.0
SS3	17.0 ± 2.5
BS1	27.0 ± 3.0
BS2	20.0 ± 2.5
BS3	15.0 ±3.0

3.4. pH Measurement

The pHs of the hydrogels were found to be in the range of 5.4 and 6.0. The results suggested that the hydrogels may be tried for topical/ transdermal application.

Table4: The pH of Hydrogels

Sample No	pH
GH	5.82 ± 0.72
CS1	5.91 ± 0.38
CS2	5.72 ± 0.44
CS3	5.64 ± 0.63
SS1	5.75 ± 0.39
SS2	5.68 ± 0.32
SS3	5.77 ± 0.63
BS1	5.41 ± 0.43
BS2	5.76 ± 0.38
BS3	5.88 ± 0.32

3.5. Hemocompatibility studies

The hemocompatibility test results of the hydrogels have been tabulated in table 5. The % hemolysis of all the samples was found to be < 5%. Hence, the hydrogels may be regarded as highly hemocompatible and hence may be tried for various biological applications [86].

Table 5: Hemocompatibility studies

Sample	% Hemolysis
GH	3.21 ± 0.21
CS1	2.15 ± 0.35
CS2	1.70 ± 0.43
CS3	1.12 ± 0.54
SS1	1.76 ± 0.31
SS2	1.24 ± 0.28
SS3	0.69 ± 0.19
BS1	1.65 ± 0.34
BS2	1.19 ± 0.32
BS3	0.64 ± 0.37

3.6. *In vitro* drug release studies

The *in vitro* drug release profiles of MZ from the hydrogels have been shown in figure 13.The rate of the release of the drug was found to be dependent on the composition of hydrogels. GH showed ~98% of the cumulative percent drug release (CPDR). Amongst the starch hydrogels, CS hydrogels have shown better release rate as compared to BS and SS hydrogels. The physical

nature of the hydrogels might have affected the CPDR of the drug from the hydrogels [86, 95]. The CPDR of MZ from the starch hydrogels was in the range of 70 % and 80 %. In general, with the increase in the starch concentration, there was a reduction in the release rate of MZ. The release of the drug from the hydrogels followed Higuchian kinetics. This indicated that the hydrogels acted as a planar matrix for the drug molecules and the release of the drug from the matrices was diffusion controlled. The Fickian value (n) was calculated from Krossmeyer-Peppas (KP) model. The 'n' values were in the range of 0.45 and 0.85 thereby suggesting a non-Fickian diffusion of the drugs from the hydrogels [96-97].

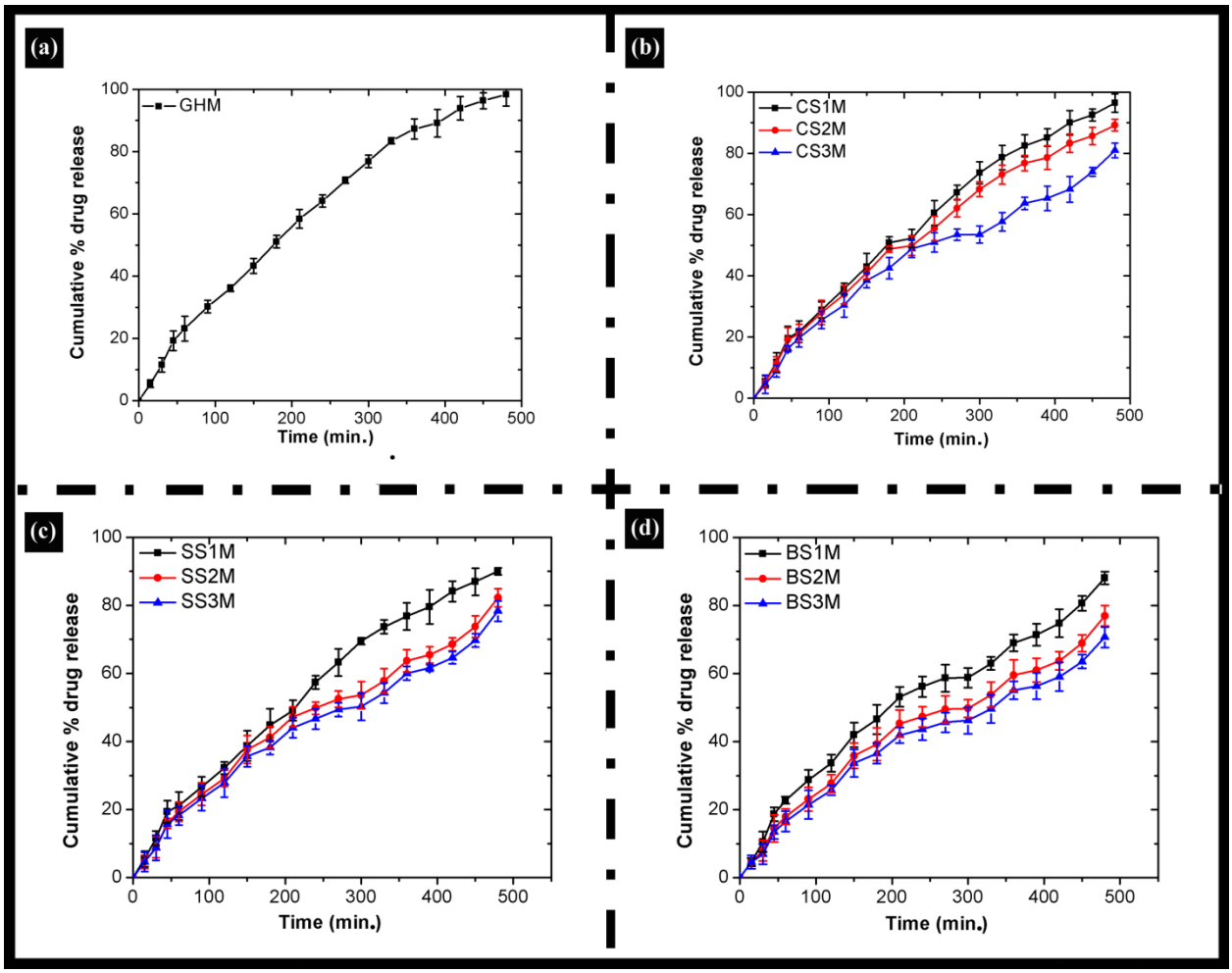

Figure 13: *In vitro* drug release profiles of (a) GHM, (b) CSM, (c) SSM and (d) BSM gels.

4. Conclusion

Gelatin-starch based physical composite hydrogels were developed by phase-separation technique. The phase separation of starch inclusions in the rich gelatin phase was confirmed by microscopic techniques. The mechanical properties of the hydrogels could be varied by altering the composition of the hydrogels. The drug loaded hydrogels showed good antimicrobial properties against both *E. coli* and *B. subtilis*. The hydrogels were found to be hemocompatible in nature. Based on the preliminary studies, the developed formulations may be tried as drug and nutraceutical delivery vehicles in pharmaceutical and food industries.

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